Novel Approaches for Identification of Broadly Cross-Reactive HIV-1 Neutralizing Human Monoclonal Antibodies and Improvement of Their Potency

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Abstract: Human monoclonal antibodies (hmAbs) that neutralize HIV isolates from different clades at physiologically relevant concentrations (broadly cross-reactive neutralizing antibodies (bcnAbs)) are rare in infected individuals. Only small number of such antibodies have been identified and extensively characterized, but efforts to elicit them *in vivo* have not been successful. We have recently developed novel approaches, based on sequential (SAP) and competitive (CAP) antigen panning methodologies, and the use of antigens with increased exposure of conserved epitopes, for enhanced identification of bcnAbs to gp120-gp41. Some of the antibodies identified by using these approaches (X5, m6, m9) bind better to gp120-CD4 complexes than to gp120 alone (CD4i antibodies); they exhibit exceptional neutralizing activity and breadth of neutralization as scFvs and on average lower potency as Fabs and IgGs. Other antibodies that compete with CD4 for binding to gp120 (m14, m18) (CD4bs antibodies) are weaker neutralizers but also exhibit broad neutralizing activity although at relatively high concentrations. The anti-gp41 antibodies (m43, m44, m45, m47 and m48) appear to have broad cross-reactivity and bind to a new group of conserved conformational epitopes distinct from those of the bcnAbs 4E10, 2F5 and Z13. Recently, the crystal structures of X5, m14 and m18 have been solved and compared to those of 17b and b12; they all contain long H3s that play a major role in their mechanism of binding. The H3s of X5, m6 and m9, unlike the others known, appear to be very flexible which may be related to the mechanism of their exceptional neutralizing activity. The further characterization of the molecular interactions of the bcnAbs with gp120-gp41 will undoubtedly help in our understanding of the mechanisms of virus neutralization, and in the design of entry inhibitors and vaccines.

Key Words: Antibodies, HIV, AIDS, vaccines, inhibitors, gp120, gp41.

INTRODUCTION

More than two decades after the discovery of HIV the development of vaccine remains a fundamental challenge to our ability to design an effective immunogen [1]. One obstacle to the development of an effective HIV vaccine has been the difficulty in inducing potent broadly cross-reactive neutralizing antibodies (bcnAbs) with protective functions against primary isolates. Defining epitopes and designing immunogens that will induce these antibodies is one of the major challenges that currently confronts the HIV vaccine field, and new approaches for development of bcnAbs, e.g. retrovaccinology [2], are currently being tested. HIV uses various strategies to evade host immune surveillance - it rapidly mutates and "hides" conserved epitopes of its envelope glycoprotein (Env) by using variable loops, heavy glycosylation, oligomerization and conformational masking [3-6]. As a result, elicitation of bcnAbs *in vivo* is rare [7] and usually occurs after relatively long periods of maturation during which the rapid generation of mutants outpaces the development of neutralizing antibodies. Identification and characterization of bcnAbs, therefore, may provide insights into the closely guarded conserved structures that could serve as epitopes for neutralizing antibodies, and has implications for development of vaccines, design of entry inhibitors as well as for understanding mechanisms for HIV entry and evasion of immune responses.

Despite extensive research efforts only several human monoclonal antibodies (hmAbs) have been identified that exhibit potent and broad HIV-1 neutralizing activity *in vitro*, and can prevent HIV-1 infection in animal models [2,8,9]. A recent clinical trial suggested that two of these bcnAbs, 2F5 and 2G12, are without side effects in humans [10,11]. However, the potency of 2F5 and 2G12 used in combination in this clinical trial was significantly lower than that of currently used HAART regimens and relapses did occur [11]. Attempts to develop immunogens that can elicit these and other bcnAbs specific to gp120 (b12) or gp41 (2F5, 4E10, Z13) have not been successful. All three known gp41-specific bcnAbs (2F5, 4E10, Z13) bind peptides from the gp41 membrane-proximal external region (MPER). In spite of the large amount of work, however, immunogens based on these peptides failed to elicit nAbs against primary isolates. Recent investigation on this revealed that the two most broadly reactive HIV-1 envelope gp41 human monoclonal antibodies (MAbs), 2F5, and 4E10, are polyspecific autoantibodies reactive with the phospholipid, cardiolipin, suggesting the autoantigen mimicry of the conserved membrane proximal epitopes of the virus; thus, current HIV-1 vaccines

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may not induce these types of antibodies [12]. Therefore, development of new neutralizing hmAbs and further increase in the potency of the currently available bcnAbs may help in the development of better approaches for prevention and treatment of HIV-1 infection. A major goal of our group has been to develop novel potent bcnAbs as potential therapeutics, and characterize their epitopes as potential vaccine immunogens, targets for inhibitors and entry intermediates. Here we review the new approaches we have recently developed for identification of such bcnAbs and improvement of their HIV-neutralizing activity, and describe several representative antibodies.

IDENTIFICATION OF HIV-NEUTRALIZING HUMAN MONOCLONAL ANTIBODIES – MAJOR APPROACHES

Two major methodologies have been used for identification of novel HIV neutralizing human monoclonal antibodies. The first approach is based on the immortalization of B lymphocytes from HIV-infected patients by EBV transformation [13,14] (the EBV transformants can also be fused with heteromyeloma cells [15-19]) followed by screening of their supernatants for antigen-specific antibodies. The second major approach which is currently gaining popularity is based on the use of phage display methodology [20] for antibody selection from phage-displayed human antibody libraries using one antigen [21] or sequentially panning against several antigens [22]. The bcnAbs 2F5 [23], 4E10 [24], 447-52D [25] and 2G12 [26-28] were obtained by immortalization of B lymphocytes from HIV-infected patients, and the bcnAbs b12 [29,30] and Z13 [31] were selected by antibody phage display. Recently, transgenic mice for human immunoglobulin genes (Xenomouse from Abgenix) in combination with traditional hybridoma technology are also being used for generation of fully human MAbs against HIV [32,33]. Our major approaches for enhanced selection of antibodies with predesigned properties include the use of three methodologies - sequential antigen panning (SAP) [22], competitive antigen panning (CAP) [Zhang *et al*. submitted] and binding kinetics modulation panning (BKMP) [Choudhry *et al*. in preparation], and three different types of antigens based on soluble ectodomains of HIV-1 envelope glycoproteins (Envs) (gp140s) - Env-receptor complexes [34], engineered Envs [35] and native Envs with enhanced exposure of conserved structures [Choudhry *et al*. in preparation]. For antibody selection we have been using an immune antibody library derived from three selected long-term nonprogressors whose sera exhibited very broad and potent HIV-1 neutralizing activity [22,36], and a large naïve antibody library derived from ten healthy individuals containing about 10^{10} different antibodies [Zhu and Dimitrov, in preparation]. Representative antibodies obtained by these approaches are shown in Table **1** and will be discussed below.

COMPLEXES OF ENVS WITH RECEPTOR MOLECULES AS ANTIGENS FOR SELECTION OF MONOCLONAL ANTIBODIES

The Env undergoes a series of conformational changes after binding receptor molecules resulting in virus entry into the host cells. We and others have hypothesized that conserved epitopes are exposed during the entry process [37-41]. To identify such conserved epitopes, we prepared Env-$gp120_{JRFL}$-CD4-CCR5 (Fig. (**1**)) [42] and used them for screening of human antibody phage libraries that resulted in the selection of a hmAb Fab, designated X5 [34].

X5 binds to gp120s and gp140s from primary isolates with an affinity in the nM range that is significantly increased in presence of CD4, indicating that X5 is a so-called CD4-induced (CD4i) antibody (Fig. (**2**)). Fab X5 exhibits potent and broad neutralizing activity comparable to that of the well characterized potent broadly HIV-1 nhmAb IgG1 b12. Unlike b12, however, X5 exhibits relatively uniform neutralizing activity when tested on more than 50 primary isolates. The crystal structure of X5 demonstrated the existence of a long protruding flexible CDR3 of the heavy chain (H3) that appears to be critical for its high binding affinity (Fig. (**3**)) [43,44]. The amino acid residues forming the epitope to which X5 binds are highly conserved, which offers a possible explanation for its broad neutralizing activity.

To further improve the binding affinity of X5 without losing its cross-reactivity, we constructed an scFv X5 mutant library and selected a novel bcnAb m9 [45] by sequentially panning against Env-receptor complexes $gp140_{89.6}$-CD4 and $gp140_{IIIB}$-CD4; this new approach, termed sequential antigen panning (SAP), for the selection of high-affinity antibodies that bind to all antigens used for the panning and screening is detailed below. M9 was extensively tested for its binding and inhibitory activity. Its binding affinity was on average 2- to 4-fold higher with a 50-percent inhibitory concentration (IC_{50}) 2- to 10-fold lower than that of parental scFv X5. Importantly, more primary HIV-1 isolates from different subtypes were neutralized by m9 than by scFv X5. Thus, both the potency and breadth of neutralization were improved. M9 neutralized more than 50 primary isolates from different HIV-1 genetic subtypes including clade C, which is the

Table 1. Summary of Representative Anti-HIV-1 bcnAbs Selected by the Novel Approaches

Antibody (ref.)	Antibody format of highest activity	Antigen used for panning	Panning methodology	Potency	Breadth	Epitope
X5 [34]	scFv	gp120-CD4-CCR5	protein G beads	++++	++++	gp120 (CD4i)
m9 [45]	scFv	gp140s-sCD4	SAP	+++++	+++++	gp120 (CD4i)
m14 [51]	IgG1	gp140s	SAP	+++	++	gp120 (CD4bs)
m43-48 [submitted]	unknown	gp140s	CAP	+++	+++	gp41

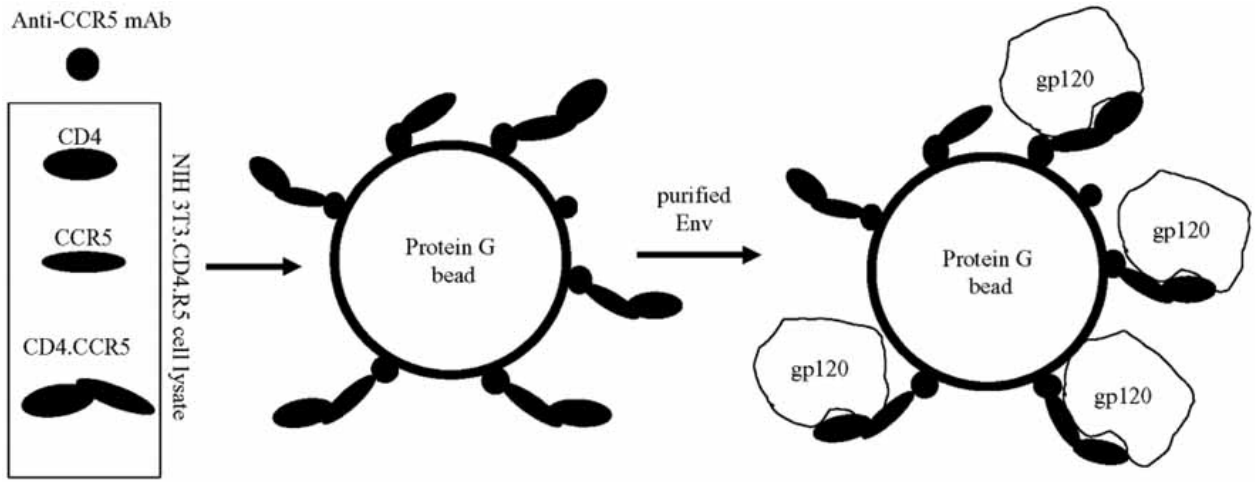

Fig. (1). Schematic illustration of purified immobilized Env-CD4-CCR5 complex preparation. Membrane-associated CD4-CCR5 complexes were immunoprecipitated with an anti-CCR5 mAb and captured with protein G beads. Purified gp120 was added and the beads were extensively washed and used for panning.

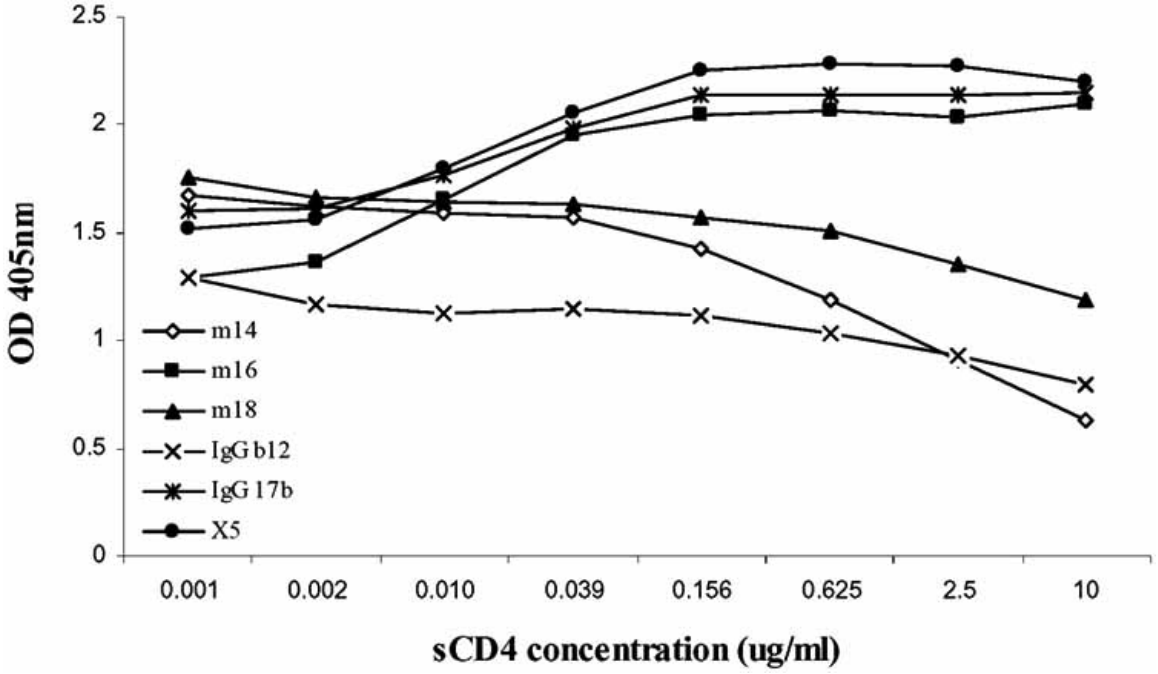

Fig. (2). Effect of sCD4 on antibody binding to gp120. $Gp120_{JRFL}$ was captured by sheep anti-gp120 polyclonal antibody (D7324) coated on the 96-well ELISA plates. Four-fold serially diluted sCD4 were simultaneously added to the wells with antibodies at a concentration which leads to 70% maximum binding. Bound antibodies were revealed by horse radish peroxidase (HRP) conjugated anti-human IgG, F(ab')2 as second antibody and ABTS as substrate. Optical density at 405nm was measured after color development for five minutes.

dominant subtype around the world, and clade B, which is dominant in the United States. To date, only several other potent broadly HIV-1 neutralizing hmAbs (b12, 2G12, 447-52D, X5, 2F5, Z13, and 4E10) are known of the large number of antibodies tested, and it appears that m9 exhibits exceptional potency and breadth of neutralization. For example, m9 was superior to any other antibody when tested against a panel of 17 clade C primary isolates [Montefiori *et al*. in preparation]. M9 neutralized 15 isolates with IC_{50}<50 μg/ml, ten out of which with IC_{50}<2 μg/ml. The median IC_{50} for the whole panel of isolates was less than 2 μg/ml. Previously, soluble CD4 (sCD4), IgG1 b12, 2G12 and 2F5 were also tested with the same panel of 16 clade C isolates (TV 1 was excluded) - sCD4 neutralized one, 2F5 neutralized two isolates with IC_{80}<50 μg/ml, 2G12 neutralized none, and IgG1 b12 neutralized nine isolates with IC_{80}<50 μg/ml with a median IC_{80} of about 50 μg/ml [46].

Alanine scanning mutagenesis suggested that the m9 epitope overlaps with the Fab X5 epitope. The increased affinity and potency may be ascribed to the changes in the conformation of the H3 and H2. M9 differs from scFv X5 by three mutations: S181T (H2), D229G (H3) and T251N. Molecular dynamics simulations, based on the crystal struc-

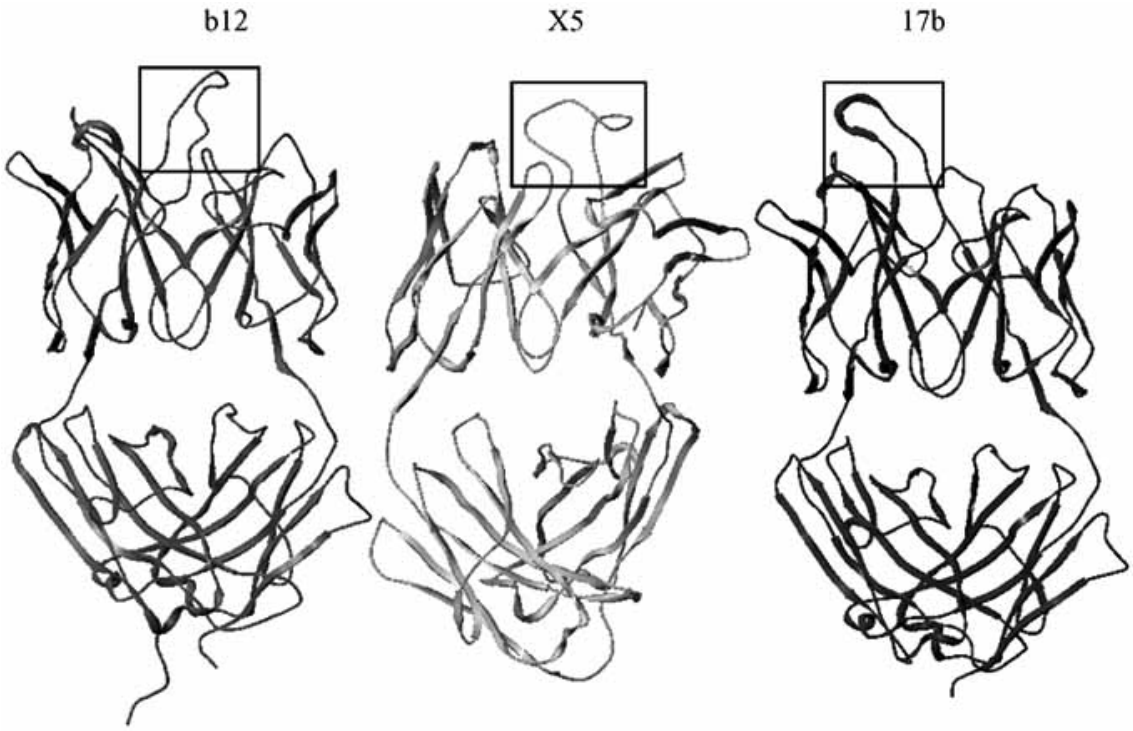

Fig. (3). Long protruding H3 loops observed in the crystal structures of b12, X5 and 17b.

ture of Fab X5 as a template for homologous modeling of the structures of scFv X5 and m9, predicted that the D229G mutation and changes in the orientation of the two residues (W227 and Y233) that are critical for X5 binding, can lead to an increase in the flexibility of the H3 loop and higher affinity of binding to gp120-CD4 complexes.

The molecular mechanisms that determine the antibody potency and breadth of HIV-1 neutralization for CD4bs antibodies depend primarily on their binding affinity to the native envelope glycoprotein (Env) before its interaction with receptor molecules. However, other hmAbs including 2F5, Fab X5 and its derivative scFv forms, m6 and m9, appear to exhibit a post-CD4 binding mechanism of neutralization that may be dependent on the lifetime of entry intermediates. Thus, we hypothesized that rate constants of binding of such CD4i antibodies would be an important determinant of their neutralization activity. To test this hypothesis we began to measure the on and off rate constants of CD4i HIV-1 neutralizing hmAbs to gp120s and gp140s by using Biacore in combination with virus neutralization assays [Choudhry *et al*. in preparation]. We observed higher association binding rate constant (k_{on}) and affinity (2- and 10-fold higher) for scFvs m9 than for Fab X5 for several Envs that correlated with the antibody inhibitory activities. Our kinetic and neutralization data also support the observation that for several primary isolates neutralization by CD4i antibodies could be affected by their size [47].

Based on these observations, we developed a new approach for selection of CD4i scFv antibodies with improved k_{on} by using Biacore [Choudhry *et al*. in preparation]. M99 was selected from the scFv X5 mutant library using this approach and it showed enhanced kinetics of interaction with different Env glycoproteins (ten-fold higher k_{on}) and increased neutralizing activity for different primary isolates compared to scFv X5. The improved k_{on} and affinity of m99 also correlate with its increased neutralizing potency. These findings may assist in the design of vaccines and entry inhibitors for HIV-1. Interestingly, m99 and m9 have differential neutralization profiles suggesting their potential use in combination for HIV-1 neutralization.

M9 and m99 alone or in combination, or as fusion proteins with sCD4 or immunotoxin (e.g., PE) have potential as therapeutics for HIV-1 infection. One concern is the short half-life of scFvs *in vivo* compared to IgGs. To extend the scFv *in vivo* half-life, the scFvs could be PEGylated. Recently, CD4i antibodies were found to be also potent in neutralizing HIV-2 which is another demonstration of the broad neutralizing activity and high conservation of their epitopes [48].

TETHERED AND NATIVE ENVS WITH ENHANCED EXPOSURE OF CONSERVED EPITOPES AS ANTIGENS

Binding of the Env complex with CD4 to coreceptor molecules initiates a series of conformational changes that are the heart of the fusion machinery driving viral entry. We hypothesized that intermediate Env conformations at different stages of the entry process could be exhibited in tethered Envs where gp120 and the ectodomain of gp41 are joined by flexible linkers of different lengths (Fig. (**4**)) [35]. We further hypothesized that those "frozen" transient intermediate conformations can expose conserved epitopes during the fusion process suggesting a novel approach for design of recombinant Envs as antigens for selection of bcnAbs and as potential immnunogens. The results showed that tethered $gp140_{89.6}$ with long flexible linkers (15 AA or 26 AA) were

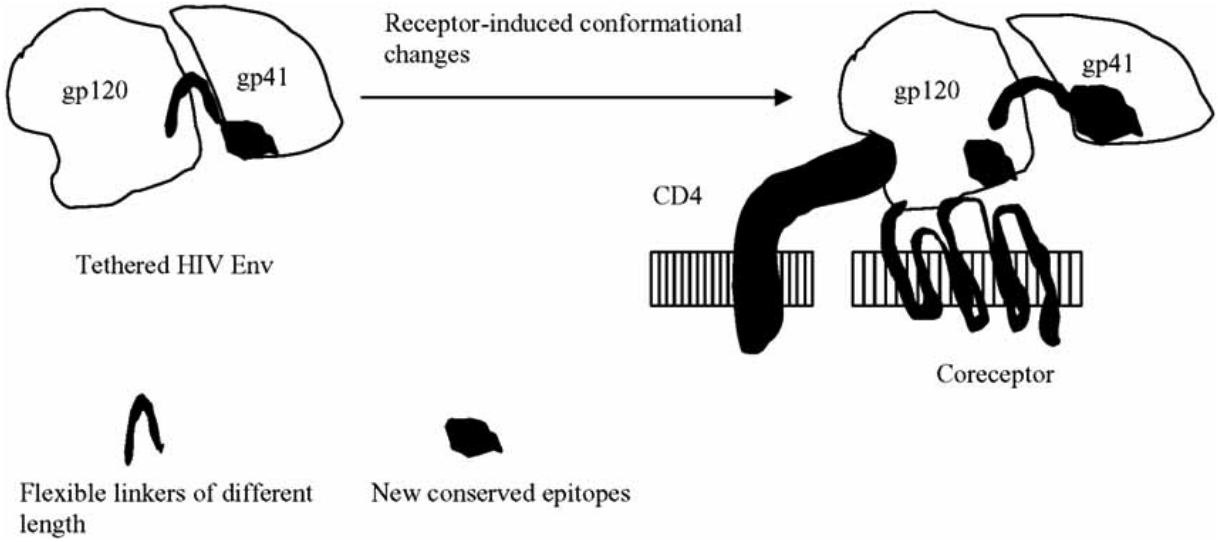

Fig. (4). Schematic illustration of a soluble tethered Env undergoing conformational changes after interaction with receptor (CD4) and coreceptor (CCR5 or CXCR4) molecules. Flexible linkers of various length stabilize the Env and constrain the conformational changes to certain intermediates that expose conserved epitopes.

stable and recognized by known HIV-1 bcnAbs, IgG1 b12, 2G12 and Fab X5, and other conformational dependent anti-gp120 and anti-gp41 monoclonal antibodies. Interestingly, compared to gp140, tethered Envs with long flexible linkers exhibited increased binding to anti-gp41 cluster II mAbs but not to cluster I mAbs. Surprisingly, these tethered Env proteins with long linkers potently inhibited membrane fusion mediated by R5, X4, and R5X4 Envs with 5-100-fold lower IC_{50} (with IC_{50} in the range from 0.3 to 3 nM in dependence on the experimental system used) than a tethered Env with short linker (4 AA), gp120, gp140, sCD4, or DP178 (T20). Cluster II mAbs but not cluster I, IV, or V mAbs can reverse the inhibitory effect of Envs with long linkers suggesting the existence of conserved gp41 structures that are important for HIV-1 entry and that can be stably exposed in the native environment of the Env even in the absence of receptor-mediated activation. Thus, tethered Envs with long linkers may be important for elucidation of viral entry mechanisms and development of novel vaccine immunogens.

We used tethered $gp140_{89.6}$ with long flexible linker as antigen for selection of anti-gp41 neutralizing hmAbs. Compared to non-tethered Envs, tethered $gp140_{89.6}$ facilitated significantly more efficient selection of anti-gp41 cross-reactive HIV-1 neutralizing antibodies by using the CAP methodology (see further discussions below).

We have also used a soluble Env ($gp140_{R2}$) isolated from a donor (R2) with nonprogressive HIV-1 infection whose serum contains broadly cross-reactive primary isolate neutralizing antibodies (bcnAbs); its envelope glycoprotein (Env) is competent for CD4-independent infection and a soluble oligomeric form ($gp140_{R2}$) elicited bcnAbs in monkeys indicating enhanced exposure of conserved epitopes [49-51]. This is why we hypothesized that panning of phage-displayed antibody libraries against ($gp140_{R2}$) is likely to result in selection of bcnAbs. To test this hypothesis, and to begin to identify and characterize conserved epitopes on R2 we used $gp140_{R2}$ as an antigen for panning of an immune human antibody library derived from three long-term non-progressors with high level of bcnAbs. Interestingly, we selected antibodies (m22 and m24), which are almost identical to the bcnAbs m18 and m14 (see below), and also three anti-gp41 cross-reactive antibodies described below (m44, m45, m46) [Zhang *et al*. submitted; Choudhry *et al*. in preparation]. These results suggest that $gp140_{R2}$ exposes antigenically conserved epitopes that can be used for selection of broadly neutralizing antibodies, and may have implications for development of therapeutics and vaccines.

SEQUENTIAL ANTIGEN PANNING (SAP) FOR SELECTION OF CROSS-REACTIVE ANTIBODIES

The Env is highly variable, especially its variable loops including the V3 loop which is also very immunogenic and antigenic. Infection with HIV-1, immunization with Envs and panning of antibody phage-displayed libraries against the Env usually results in isolate-specific antibodies, and only in rare cases some individuals contain physiologically relevant concentrations of bcnAbs. To enhance the selection of such antibodies that recognize conserved epitopes, we developed an approach based on sequentially changing antigens during antibody selection - termed Sequential Antigen Panning (SAP) (Fig. (**5**)). Several Envs representing different viral isolates are sequentially changed during the panning and screening which leads to the selection of antibodies against epitopes shared among these antigens. By using SAP against oligomeric $gp140_{89.6}$ and $gp140_{IIIB}$ followed by screening with the same antigens and $gp120_{JRFL}$, we identified the broadly cross-reactive CD4 binding site (CD4bs) antibodies m14 [52] and m18 [22].

Fab m14 neutralizes a range of primary HIV-1 isolates from different clades. Importantly, the m14 neutralization profile is different from that of the IgG1 b12 and possibly from other HIV-1 neutralizing antibodies. The ability of m14 to potently neutralize representative isolates from clades A, B, C and F suggests that it can be used in combination with

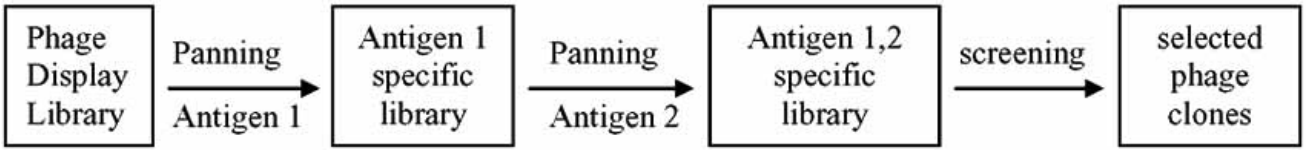

Fig. (5). Schematic representation of the sequential antigen panning (SAP) methodology. Antibody phage display library was sequentially panned against antigen 1 and 2, and the panned libraries screened for clones binding to antigen 1, 2 and other antigens. The selected phage clones bind to all antigens used for panning and screening.

other hmAbs that have poor neutralization activity or lack activity against such isolates. Because m14 competes with CD4 for binding to gp120 it is likely that its mechanism of neutralization involves interactions with Envs before binding to CD4 (Fig. (**2**)). Thus restricted access effects due to size are unlikely to play a role. To further improve the potency of Fab m14 we constructed a whole antibody molecule, IgG1 m14, which exhibited significantly higher neutralizing activity. For the same panel of clade C isolates described above, IgG1 m14 neutralized seven isolates with IC_{50}<50 μg/ml and the median IC_{50} for the whole panel of isolates was about 60 μg/ml. These results suggest that m14 could neutralize a broad range of HIV-1 primary isolates *in vitro*, and the IgG1 format would ensure long half-life and biological effector functions.

Fab m14 binds to Envs from a number of primary isolates with high affinities. Alanine scanning mutagenesis suggested that most of the residues involved in binding to gp120 are conserved, which may explain the broadly cross-reactive neutralization activity of m14. Interestingly, alanine scanning mutagenesis demonstrated the existence of some gp120 mutations and loop deletion variants that result in a significant increase of m14 binding to gp120. These gp120 mutants and loop deletion variants may provide a clue in engineering gp120 for vaccine development. The molecular mechanism of the high-affinity binding of Fab m14 to gp120 is currently under investigation. The crystal structure showed a long protruding CDR H3 loop [Darbha *et al.* in preparation], similar to the long CDR H3 of b12 that is likely to play a critical role in neutralization.

M18 is another CD4bs antibody (Fig. (**2**)) identified by using SAP. It neutralized HIV-1 primary isolates, but on average weaker than m14. Fab m18 has very high affinity for gp140s from primary isolates. For gp140s from eleven primary isolates tested, Fab m18 has EC_{50} ranging from 0.25 to 1.17 nM for ten gp140s [22]. The recently solved crystal structure of Fab m18 revealed that m18 CDR H3 loop strikingly mimics the CDR2-like loop in CD4 [53]. Docking analysis showed that the Phe 99 in the H3 of m18 plays similar role as Phe 43 of the CDR2-like loop of CD4 in binding to the gp120 Phe-cavity [53]. Blocking receptor binding site is a practical approach for blocking viral infection. Soluble CD4 (sCD4) is effective in neutralizing laboratory-adapted HIV-1 isolates, but is not effective against primary isolates. Recently, after overcoming major problems, a CD4 mimics was designed that closely resembles the structure and function of CD4 [54]. A potential problem with CD4 mimics and to some extent sCD4 is the possibility for immune responses although CD4 is a human protein but still modified. As a human mAb, m18 may not be immunogenic and the design of new entry inhibitors based on its H3 loop appears a potentially fruitful approach.

Considering that CD4 binding triggers gp120 conformational changes to expose conserved epitopes, we also did SAP against $gp140_{89.6}$-CD4 and $gp140_{IIIB}$-CD4 complexes and identified two CD4i antibodies m16 [55] and m12 [Zhang *et al*. in preparation]. The affinity of m16 for gp120-CD4 is on average ten-fold higher than for gp120 alone (Fig. (**2**)). Unlike X5, IgG1 m16 didn't show significant size restrictions in neutralization which is usually observed with CD4i antibodies. m12 appeared to be a domain antibody (lack of light chain) and it showed inhibitory activity in cell-cell fusion. With the half size of a Fab, m12 has advantages over other CD4i antibodies targeting conserved coreceptor binding site as an entry inhibitor. We are currently improving m12 affinity and solubility by introducing random mutagenesis and selection against gp140-CD4 complexes.

COMPETITIVE ANTIGEN PANNING FOR SELECTION OF ANTI-GP41 ANTIBODIES

A number of anti-gp41 hmAbs have been identified and characterized but only a minority are neutralizing including 2F5, 4E10 obtained from immortalized B cells and Fab Z13 obtained from phage display panning against a gp41 peptide and HIV-1 MN virion [56]. All three anti-gp41 bcnAbs recognize linear peptides on gp41, but it appears that these peptides used as immunogens do not lead to elicitation of antibodies neutralizing primary isolates. For example, the use of ELDKWA inserted into a carrier protein did not induce HIV-1 neutralizing antibodies [57] likely due to lack of appropriate environment, including the lipid of the viral membrane, to support the structure of this peptide in the context of gp41 [58], or due to the autoantigen mimicry nature of the conserved membrane proximal epitopes of gp41 as mentioned above [12]. Identification of novel anti-gp41 bcnAbs could provide new ideas for design of vaccine immunogens based on gp41.

The Env gp41 is genetically more conserved than gp120 and could contain many conserved epitopes, but typically phage library panning against soluble Envs (gp140s) results in the selection of anti-gp120 antibodies. To identify cross-reactive antibodies that may bind to conserved gp41 structures with potential as HIV vaccine immunogens, we have developed a competitive antigen panning (CAP) methodology. CAP is based on the idea to select Env-interacting antibodies in the presence of excess gp120, which facilitates the identification of gp41-reactive antibodies in the context of native Env (Fig. (**6**)). The CAP resulted in selection of a significant number of phage-displayed antibodies that bound gp140 but did not bind gp120 (gp41 binders). The tether

$gp140_{89.6}$ described above and non-tethered $gp140_{R2}$ and $gp140_{CM243}$ were used as antigens. The tethered $gp140_{89.6}$ was the most effective antigen among those three tested in the selection of anti-gp41 bcnAbs [Zhang *et al*. submitted].

By using CAP, we selected five antibodies (m43, m44, m45, m47 and m48) from an immune human antibody phage library derived from long-term nonprogressors with high concentrations of broadly nAbs. The newly identified anti-gp41 antibodies were tested in a pseudovirus-based assay and in a peripheral blood mononuclear cell (PBMC)-based assay that may resemble more closely the *in vivo* infection. M43, 44, 45, 47, 48 exhibited neutralizing activity against a panel of primary isolates from different clades with a potency on average comparable to that of Fab Z13 and IgG1 4E10. They bound with high affinity to soluble Envs (gp140s) from primary isolates including $gp140_{89.6}$, $gp140_{R2}$ and $gp140_{CM243}$ that were used for their selection. Their cross-reactivity is likely to be related to their broad neutralizing activity. We have also found that the neutralizing activity of these antibodies is dependent on their format. It appears that on average the neutralizing activity of the anti-gp41 Fabs is somewhat better than that of the respective IgGs. However, the difference is not as significant as for some of the CD4i antibodies. The difference was isolate-dependent and for some isolates the neutralizing activity of the IgGs was higher than that of the Fabs.

The epitopes of the three known anti-gp41 bcnAbs 2F5, 4E10 and Z13 are localized in the membrane-proximal external region (MPER) of gp41 and include stretches of known sequences; they do bind to peptides containing these sequences and denatured gp140 [31]. In contrast to 2F5 and 4E10/Z13 our newly selected anti-gp41 antibodies did not bind denatured gp140. Competition with known anti-gp41 antibodies and lack of binding to antigen mapping gp41 peptides also suggest that the newly identified anti-gp41 antibodies recognize conformational epitopes. Further characterization indicated that disulfide bonds in gp41 are important for the structural integrity of their epitopes. Our findings suggest the existence of a new group of conformational neutralization epitopes on gp41. These conserved gp41 structures may have potential as HIV vaccine immunogens and as targets for therapeutics.

Future structural and mutagenesis studies are required for more complete characterization of these epitopes to help foster the design of immunogens able to elicit broadly HIV-1 neutralizing antibodies. The newly identified anti-gp41 antibodies could also have some potential as a component of a combination therapy regimen. Their neutralizing activity is not as high as that of some anti-gp120 antibodies, e.g. m9, and this has also been found for other anti-gp41 antibodies when compared to the anti-gp120 antibody b12 [59]. However, the anti-gp41 antibodies exhibit relatively broad neutralizing activity that could help to prevent emergence of neutralization resistant mutants. Further studies are also required for elucidation of the mechanism of neutralization by the newly identified anti-gp41 antibodies. Crystallization of these antibodies is in progress.

CONCLUSIONS

The development of novel approaches for identification of bcnAbs and their conserved epitopes, including novel antigens and methodologies for selection of such antibodies,

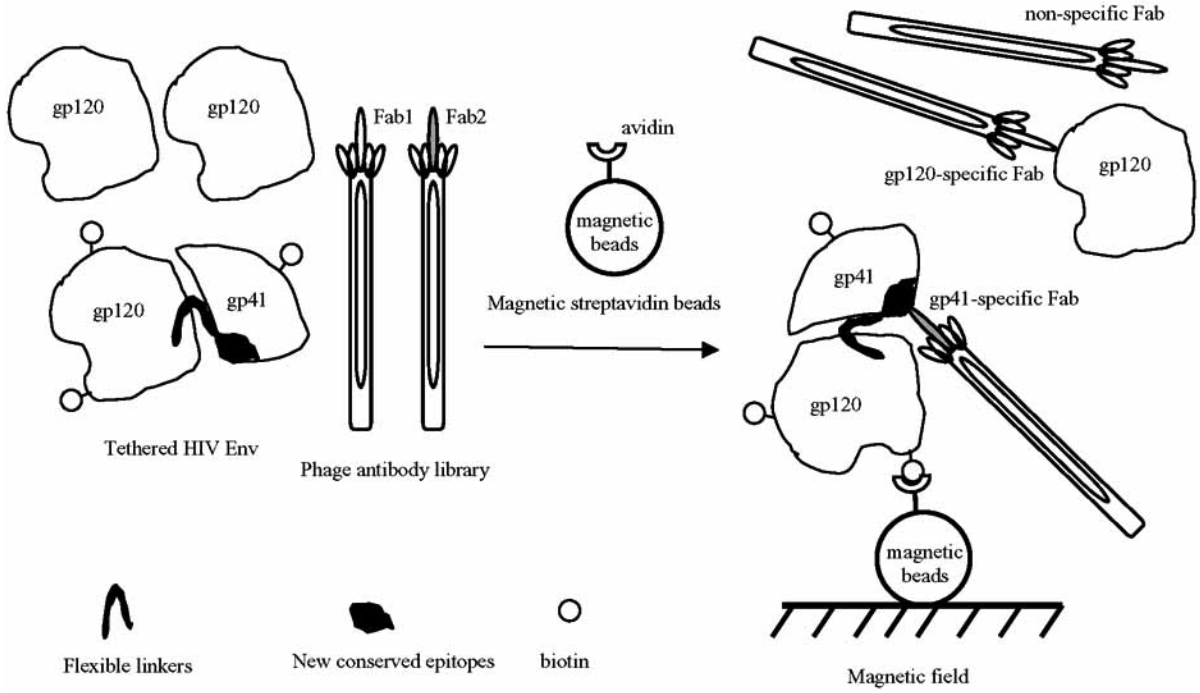

Fig. (6). Schematic illustration of the competitive antigen panning (CAP) methodology. Biotinylated tethered gp140 mixed with 5-10 fold higher molar concentrations of non-biotinylated gp120 are used for panning against phage libraries. The complexes of anti-gp41 phage antibodies with tethered gp140 were separated from the panning mixture by using magnetic streptavidin conjugated beads and a magnetic field. Bound phage were eluted, amplified and used for subsequent panning.

undoubtedly will contribute to our understanding of the mechanisms of HIV neutralization and evasion of immune responses, which in turn could help in the design of new inhibitors and vaccine immunogens. During the last several years we and others have developed such approaches and identified a number of novel bcnAbs directed to three major regions of the Env that contain a number of potentially important conserved and immunogenic epitopes defined by their binding to CD4, coreceptors and our recently identified anti-gp41 antibodies. The region with enhanced exposure of epitopes after CD4 binding appears to be highly conserved and the antibodies that target those epitopes exhibit exceptional potency and breadth of neutralization. However, a major obstacle for the use of these antibodies as HIV inhibitors *in vivo* is the limitations imposed by their size – an increase in their half-life and even further increase in their potency could help in development of potentially clinically useful inhibitors. A challenging problem is to develop a vaccine based on these epitopes – if successful such a vaccine could lead to elicitation of very potent antibodies with a broad neutralizing activity. The potency of CD4bs antibodies can also be high but only for restricted set of isolates and resistance can develop very quickly. A combination of such antibodies could have potential as HIV inhibitors and a vaccine designed to elicit such combinations could be a valuable strategy for vaccine design. The potency of the known anti-gp41 antibodies is on average relatively weak but they have broad neutralizing activity. Thus their potential as inhibitors is in combination. The identification of the new anti-gp41 antibodies offers some hope for the design of novel vaccine immunogens that could elicit bcnAbs *in vivo.*

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ABBREVIATIONS

bcnAbs = Broadly cross - reactive neutralizing antibodies

hmAbs = Human monoclonal antibodies

nAb = Neutralizing antibodies

scFv = Single-chain variable fragment

Env = Envelope glycoprotein

sCD4 = Soluble CD4

CD4bs = CD4 binding site antibodies

CD4i = CD4 induced antibodies

SAP = Sequential antigen panning

CAP = Competitive antigen panning

PBMC = Peripheral blood mononuclear cell

MPER = Membrane-proximal external region

HAART= Highly active antiretroviral therapy

REFERENCES

References 60-62 are related articles recently published in Current Pharmaceutical Design.

[1] McMichael AJ, Hanke T. HIV vaccines 1983-2003. Nat Med 2003; 9: 874-880.

[2] Burton DR. Antibodies, viruses and vaccines. Nat Rev Immunol 2002; 2: 706-713.

[3] Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, *et al*. Antibody neutralization and escape by HIV-1. Nature 2003; 422: 307-312.

[4] Richman DD, Wrin T, Little SJ, Petropoulos CJ. Rapid evolution of the neutralizing antibody response to HIV type 1 infection. Proc Natl Acad Sci USA 2003; 100: 4144-4149.

[5] Garber DA, Silvestri G, Feinberg MB. Prospects for an AIDS vaccine: three big questions, no easy answers. Lancet Infect Dis 2004; 4: 397-413.

[6] Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, *et al.* HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature 2002; 420: 678-682.

[7] Burton DR, Montefiori DC. The antibody response in HIV-1 infection. AIDS 1997; 11(Suppl A): S87-S98.

[8] Ferrantelli F, Ruprecht RM. Neutralizing antibodies against HIV -- back in the major leagues? Curr Opin Immunol 2002; 14: 495-502.

[9] Veazey RS, Shattock RJ, Pope M, Kirijan JC, Jones J, Hu Q, *et al*. Prevention of virus transmission to macaque monkeys by a vaginally applied monoclonal antibody to HIV-1 gp120. Nat Med 2003; 9: 343-346.

[10] Armbruster C, Stiegler GM, Vcelar BA, Jager W, Michael NL, Vetter N, *et al*. A phase I trial with two human monoclonal antibodies (hMAb 2F5, 2G12) against HIV-1. AIDS 2002; 16: 227-233.

[11] Stiegler G, Armbruster C, Vcelar B, Stoiber H, Kunert R, Michael NL, *et al*. Antiviral activity of the neutralizing antibodies 2F5 and 2G12 in asymptomatic HIV-1-infected humans: a phase I evaluation. AIDS 2002; 16: 2019-2025.

[12] Haynes BF, Fleming J, St Clair WE, Katinger H, Stiegler G, Kunert R, *et al*. Cardiolipin Polyspecific Autoreactivity in Two Broadly Neutralizing HIV-1 Antibodies. Science 2005; 308: 1906-8.

[13] Gorny MK, Gianakakos V, Sharpe S, Zolla-Pazner S. Generation of human monoclonal antibodies to human immunodeficiency virus. Proc Natl Acad Sci USA 1989; 86: 1624-1628.

[14] Robinson JE, Holton D, Pacheco-Morell S, Liu J, McMurdo H. Identification of conserved and variant epitopes of human immunodeficiency virus type 1 (HIV-1) gp120 by human monoclonal antibodies produced by EBV-transformed cell lines. AIDS Res Hum Retroviruses 1990; 6: 567-579.

[15] Grunow R, Jahn S, Porstmann T, Kiessig SS, Steinkellner H, Steindl F, *et al*. The high efficiency, human B cell immortalizing heteromyeloma CB-F7. Production of human monoclonal antibodies to human immunodeficiency virus. J Immunol Methods 1988; 106: 257-265.

[16] Buchacher A, Predl R, Strutzenberger K, Steinfellner W, Trkola A, Purtscher M, *et al*. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res Hum Retroviruses 1994; 10: 359-369.

[17] Gorny MK, Xu JY, Gianakakos V, Karwowska S, Williams C, Sheppard HW, *et al*. Production of site-selected neutralizing human monoclonal antibodies against the third variable domain of the human immunodeficiency virus type 1 envelope glycoprotein. Proc Natl Acad Sci USA 1991; 88: 3238-3242.

[18] Posner MR, Elboim H, Santos D. The construction and use of a human-mouse myeloma analogue suitable for the routine production of hybridomas secreting human monoclonal antibodies. Hybridoma 1987; 6: 611-625.

[19] Posner MR, Hideshima T, Cannon T, Mukherjee M, Mayer KH, Byrn RA. An IgG human monoclonal antibody that reacts with HIV-1/GP120, inhibits virus binding to cells, and neutralizes infection. J Immunol 1991; 146: 4325-4332.

[20] Barbas CF, Burton DR, Scott JK, Silverman GJ. Phage Display: A Laboratory Mannual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 2001.

[21] Burton DR, Barbas CF, Persson MA, Koenig S, Chanock RM, Lerner RA. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc Natl Acad Sci USA 1991; 88: 10134-10137.

[22] Zhang MY, Shu Y, Phogat S, Xiao X, Cham F, Bouma P, *et al*. Broadly cross-reactive HIV neutralizing human monoclonal antibody Fab selected by sequential antigen panning of a phage display library. J Immunol Methods 2003; 283: 17-25.

[23] Muster T, Steindl F, Purtscher M, Trkola A, Klima A, Himmler G, *et al*. A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1. J Virol 1993; 67: 6642-6647.

[24] Stiegler G, Kunert R, Purtscher M, Wolbank S, Voglauer R, Steindl F, *et al*. A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1. AIDS Res Hum Retroviruses 2001; 17: 1757-1765.

[25] Gorny MK, Conley AJ, Karwowska S, Buchbinder A, Xu JY, Emini EA, *et al*. Neutralization of diverse human immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. J Virol 1992; 66: 7538-7542.

[26] Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO, *et al*. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J Virol 2002; 76: 7293-7305.

[27] Scanlan CN, Pantophlet R, Wormald MR, Ollmann SE, Stanfield R, Wilson IA, *et al*. The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. J Virol 2002; 76: 7306-7321.

[28] Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, *et al*. Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1. J Virol 1996; 70: 1100-1108.

[29] Burton DR, Pyati J, Koduri R, Sharp SJ, Thornton GB, Parren PW, *et al*. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 1994; 266: 1024-1027.

[30] Roben P, Moore JP, Thali M, Sodroski J, Barbas CF 3rd, Burton DR. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J Virol 1994; 68: 4821-4828.

[31] Zwick MB, Labrijn AF, Wang M, Spenlehauer C, Saphire EO, Binley JM, *et al*. Broadly neutralizing antibodies targeted to the membrane-proximal external region of human immunodeficiency virus type 1 glycoprotein gp41. J Virol 2001; 75: 10892-10905.

[32] Davis CG, Jia XC, Feng X, Haak-Frendscho M. Production of human antibodies from transgenic mice. Methods Mol Biol 2004; 248: 191-200.

[33] Okada N, Yin S, Asai S, Kimbara N, Dohi N, Hosokawa M, *et al*. Human IgM Monoclonal Antibodies Reactive with HIV-1-Infected Cells Generated Using a Trans-Chromosome Mouse. Microbiol Immunol 2005; 49: 447-459.

[34] Moulard M, Phogat SK, Shu Y, Labrijn AF, Xiao X, Binley JM, *et al*. Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes. Proc Natl Acad Sci USA 2002; 99: 6913-6918.

[35] Chow YH, Wei OL, Phogat S, Sidorov IA, Fouts TR, Broder CC, *et al*. Conserved structures exposed in HIV-1 envelope glycoproteins stabilized by flexible linkers as potent entry inhibitors and potential immunogens. Biochemistry 2002; 41: 7176-7182.

[36] Montefiori DC, Pantaleo G, Fink LM, Zhou JT, Zhou JY, Bilska M, *et al*. Neutralizing and infection-enhancing antibody responses to human immunodeficiency virus type 1 in long-term nonprogressors. J Infect Dis 1996; 173: 60-67.

[37] Dimitrov DS. Fusin--a place for HIV-1 and T4 cells to meet. Nat Med 1996; 2: 640-641.

[38] Lapham CK, Ouyang J, Chandrasekhar B, Nguyen NY, Dimitrov DS, Golding H. Evidence for cell-surface association between fusin and the CD4-gp120 complex in human cell lines. Science 1996; 274: 602-605.

[39] Jones PL, Korte T, Blumenthal R. Conformational changes in cell surface HIV-1 envelope glycoproteins are triggered by cooperation between cell surface CD4 and co-receptors. J Biol Chem 1998; 273: 404-409.

[40] Devico A, Silver A, Thronton AM, Sarngadharan MG, Pal R. Covalently crosslinked complexes of human immunodeficiency virus type 1 (HIV-1) gp120 and CD4 receptor elicit a neutralizing immune response that includes antibodies selective for primary virus isolates. Virology 1996; 218: 258-263.

[41] Fouts T, Godfrey K, Bobb K, Montefiori D, Hanson CV, Kalyanaraman VS, *et al*. Crosslinked HIV-1 envelope-CD4 receptor complexes elicit broadly cross- reactive neutralizing antibodies in rhesus macaques. Proc Natl Acad Sci USA 2002; 99: 11842-11847.

[42] Xiao X, Phogat S, Shu Y, Phogat A, Chow YH, Wei OL, *et al*. Purified complexes of HIV-1 envelope glycoproteins with CD4 and CCR5(CXCR4): production, characterization and immunogenicity. Vaccine 2003; 21: 4275-4284.

[43] Darbha R, Phogat S, Labrijn AF, Shu Y, Gu Y, Andrykovitch M, *et al*. Crystal structure of the broadly cross-reactive HIV-1-neutralizing Fab X5 and fine mapping of its epitope. Biochemistry 2004; 43: 1410-1417.

[44] Huang CC, Tang M, Zhang MY, Majeed S, Montabana E, Stanfield RL, *et al*. Structure of a V3-containing HIV-1 gp120 core. Science 2005; 310: 1025-1028.

[45] Zhang MY, Shu Y, Rudolph D, Prabakaran P, Labrijn AF, Zwick MB, *et al*. Improved breadth and potency of an HIV-1-neutralizing human single-chain antibody by random mutagenesis and sequential antigen panning. J Mol Biol 2004; 335: 209-219.

[46] Bures R, Morris L, Williamson C, Ramjee G, Deers M, Fiscus SA, *et al*. Regional clustering of shared neutralization determinants on primary isolates of clade C human immunodeficiency virus type 1 from South Africa. J Virol 2002; 76: 2233-2244.

[47] Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M, *et al*. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. J Virol 2003; 77: 10557-10565.

[48] Decker JM, Bibollet-Ruche F, Wei X, Wang S, Levy DN, Wang W, *et al*. Antigenic conservation and immunogenicity of the HIV coreceptor binding site. J Exp Med 2005; 201: 1407-1419.

[49] Quinnan GV, Zhang PF, Fu DW, Dong M, Alter HJ. Expression and characterization of HIV type 1 envelope protein associated with a broadly reactive neutralizing antibody response. AIDS Res Hum Retroviruses 1999; 15: 561-570.

[50] Zhang PF, Bouma P, Park EJ, Margolick JB, Robinson JE, Zolla-Pazner S, *et al*. A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J Virol 2002; 76: 644-655.

[51] Quinnan GV, Jr., Yu XF, Lewis MG, Zhang PF, Sutter G, Silvera P, *et al*. Protection of rhesus monkeys against infection with minimally pathogenic simian-human immunodeficiency virus: correlations with neutralizing antibodies and cytotoxic T cells. J Virol 2005; 79: 3358-3369.

[52] Zhang MY, Xiao X, Sidorov IA, Choudhry V, Cham F, Zhang PF, *et al*. Identification and characterization of a new cross-reactive human immunodeficiency virus type 1-neutralizing human monoclonal antibody. J Virol 2004; 78: 9233-9242.

[53] Prabakaran P, Gan JH, Wu YQ, Zhang MY, Dimitrov DS, and Ji X. Structural Mimicry of CD4 by a cross-Reactive HIV-1 Neutralizing Antibody with CDR-H2 and H3 Containing Unique Motifs. J Mol Biol 2006; 357: 82-99.

[54] Martin L, Stricher F, Misse D, Sironi F, Pugniere M, Barthe P, *et al*. Rational design of a CD4 mimic that inhibits HIV-1 entry and exposes cryptic neutralization epitopes. Nat Biotechnol 2003; 21: 71-76.

[55] Zhang MY, Shu Y, Sidorov I, Dimitrov DS. Identification of a novel CD4i human monoclonal antibody Fab that neutralizes HIV-1 primary isolates from different clades. Antiviral Res 2004; 61: 161-164.

[56] Zolla-Pazner S. Identifying epitopes of HIV-1 that induce protective antibodies. Nat Rev Immunol 2004; 4: 199-210.

[57] Coeffier E, Clement JM, Cussac V, Khodaei-Boorane N, Jehanno M, Rojas M, *et al*. Antigenicity and immunogenicity of the HIV-1 gp41 epitope ELDKWA inserted into permissive sites of the MalE protein. Vaccine 2000; 19: 684-693.

[58] Ofek G, Tang M, Sambor A, Katinger H, Mascola JR, Wyatt R, *et al*. Structure and mechanistic analysis of the anti-human immunodeficiency virus type 1 antibody 2F5 in complex with its gp41 epitope. J Virol 2004; 78: 10724-10737.

[59] Binley JM, Wrin T, Korber B, Zwick MB, Wang M, Chappey C, *et al*. Comprehensive cross-clade neutralization analysis of a panel of anti-human immunodeficiency virus type 1 monoclonal antibodies. J Virol 2004; 78: 13232-13252.

[60] Agrawal L, Lu X, Jin Q, Alkhatib G. Anti-HIV therapy: Current and future directions. Curr Pharm Des 2006; 12(16): 2031-55.

[61] Bregenholt S, Jensen A, Lantto J, Hyldig S, Haurum JS. Recombinant human polyclonal antibodies: A new class of therapeutic antibodies against viral infections. Curr Pharm Des 2006; 12(16): 2007-15.

[62] Markovic I. Advances in HIV-1 entry inhibitors: strategies to interfere with receptor and coreceptor engagement. Curr Pharm Des 2006; 12(9): 1105-19.